

11/PRTS

Description**Human Fetus Chondrocyte-Derived Gene****5 Technical Field**

This invention relates to a gene expressed specifically in differentiated human chondrocytes, a protein encoded by the gene, and an antibody against the protein.

10 Background Art

No methods for monolayer culturing human chondrocytes in a differentiated state have been established. This has made it difficult to search for a gene specifically expressed in differentiated chondrocytes, and analyze the properties of a protein encoded by the gene.

15 Disclosure of the Invention

Recently, the inventor of the present invention has discovered a method of monolayer culture for human chondrocytes in a differentiated state (M. Shen, T. Kawamoto, W. Yan, K. Nakamasu, M. Tamagami, Y. Koyano, M. Noshiro, and Y. Kato, Biochemical and Biophysical Research Communications 236, 294-298 (1997)). The inventor has found that a gene specifically expressed in human chondrocytes in a differentiated state can be searched for by using such a culture method.

Based on these findings, the inventor looked for genes different in expression between human chondrocytes in a differentiated state and chondrocytes in a dedifferentiated state, and found out a gene specifically expressed in former cells. This finding led to the present invention.

That is, the invention provides a gene expressed specifically in differentiated cells (cells in various animals, especially cells including chondrocytes and human chondrocytes), a protein encoded by the gene, and an antibody against the protein. The invention also provides the gene or its fragment, nucleotide sequences complementary to them, the protein or its mutant product, fragments thereof, a kit and a method for detecting differentiated chondrocytes with the use of the antibody, or a kit and a method for screening a regulator of cell differentiation induction.

More particularly, the invention provides [1] a protein specifically expressed in differentiated chondrocytes, including an ezrin-like domain, a Db1 domain, and a pleckstrin domain. The invention also provides [2] a protein having an amino acid sequence set forth in SEQ ID NO: 2 in the sequence listing. The invention also provides [3] a protein comprising the amino acid sequence set forth in SEQ ID NO: 2 in the sequence listing in which one to several amino acids have been deleted, substituted or added, the protein being specifically expressed in differentiated chondrocytes, and the protein

being such that (1) an amino acid sequence of a portion of the protein corresponding to an amino acid sequence ranging from the 1st to 374th amino acids in SEQ ID NO: 2 in the sequence listing has homology of 85% or more to the amino acid sequence ranging from the 1st to 374th amino acids in the SEQ ID NO: 2, (2) an amino acid sequence of a portion of the protein corresponding to an amino acid sequence ranging from the 544th to 737th amino acids in SEQ ID NO: 2 in the sequence listing has homology of 85% or more to the amino acid sequence ranging from the 544th to 737th amino acids in the SEQ ID NO: 2, and (3) an amino acid sequence of a portion of the protein corresponding to an amino acid sequence ranging from the 764th to 854th amino acids in SEQ ID NO: 2 in the sequence listing has homology of 85% or more to the amino acid sequence ranging from the 764th to 854th amino acids in the SEQ ID NO: 2.

Further, the invention provides [4] DNA encoding the protein described in any one of the [1] to [3]. The invention also provides [5] a gene comprising DNA shown in the following (a) or (b): (a) DNA comprising a nucleotide sequence ranging from the 49th to 3,183rd bases in a nucleotide sequence set forth in SEQ ID NO: 1 in the sequence listing. (b) DNA which is hybridized under stringent conditions with DNA having a nucleotide sequence ranging from the 49th to 3,183rd bases in a nucleotide sequence set forth in SEQ ID NO: 1 in the sequence listing, and which encodes a protein

specifically expressed in differentiated chondrocytes. The invention also provides [6] DNA having a part of or all of a nucleotide sequence set forth in SEQ ID NO: 1 in the sequence listing, or having a nucleotide sequence complementary to them.

Further, the invention provides [7] an antibody against the protein described in any one of [1] to [3].

Further, the invention provides [8] a kit for detecting differentiated chondrocytes, characterized by containing at least one of the following (a) to (c) as an active ingredient: (a) The protein described in any one of [1] to [3]. (b) The DNA described in any one of [4] to [6]. (c) The antibody described in [7]. The invention also provides [9] a method for detecting differentiated chondrocytes, characterized by using the kit described in [8].

Further, the invention provides [10] a kit for screening a regulator of cell differentiation induction, characterized by containing at least one of the following (a) to (c) as an active ingredient: (a) The protein described in any one of [1] to [3]. (b) The DNA described in any one of [4] to [6]. (c) The antibody described in [7]. The invention also provides [11] a method for screening a regulator of cell differentiation induction, characterized by using the kit described in [10].

Further, the invention provides [12] a screening method in which the regulator of cell differentiation

induction described in [11] is an anti-tumor agent.

The amino acid sequence of the protein according to the present invention contains three domains, i.e., ezrin-like domain, Dbl homology (DH) domain, and pleckstrin homology (PH) domain, arranged in this order from the N-terminus in one molecule. Hence, this novel protein may be a very important factor for controlling the activity of an Rho family which exists near the cell membrane and regulates the function of the actin-based cytoskeleton responsible for the maintenance of cell shape and cell motility.

It becomes possible, moreover, to develop a drug for regulating cell differentiation induction or the proliferation of cancer, by activating or inhibiting the function of the protein according to the invention.

The invention also makes it possible to develop a probe indispensable for the analysis of the mechanism of cartilage differentiation or degeneration. The invention further permits the development of gene therapies of osteoarthritis and rheumatoid arthritis.

Brief Description of the Drawings

Fig. 1 is a view showing a nucleic acid nucleotide sequence of CDEP cDNA, and an amino acid sequence deduced therefrom, in which ezrin-like domain is indicated by an open frame, Dbl homology (DH) domain is underlined, pleckstrin homology (PH) domain is double-underlined, an

asterisk downstream from a protein coding region represents a stop codon, a poly(A) addition signal is indicated by a dashed line, and a poly(A) addition site is indicated by a triangle;

5 Fig. 2A is an electrophoretogram showing the results of northern blotting analysis of CDEP mRNA in various fetal human tissues, in which Ch- denotes a Bt2cAMP-untreated fibroblastic chondrocytes, Ch+ denotes a Bt2cAMP-dependent differentiated chondrocytes; Br denotes brain; He denotes heart; Li denotes liver; Sp denotes spleen; In denotes intestine; Lu denotes lung; Te denotes testis; Pl denotes placenta; Mu denotes muscle, and the size of CDEP mRNA is expressed in kb;

10 Fig. 2B is an electrophoretogram showing the results of northern blotting analysis of CDEP mRNA in various adult tissues, in which Ch- denotes a Bt2cAMP-untreated fibroblastic chondrocytes, Ch+ denotes a Bt2cAMP-dependent differentiated chondrocytes; Br denotes brain; He denotes heart; Li denotes liver; Sp denotes spleen; In denotes intestine; Lu denotes lung; Te denotes testis; Pl denotes placenta; Mu denotes muscle, and the size of CDEP mRNA is expressed in kb;

15 1K5
C2 Fig. 3 is a view showing the arrangement of an ezrin-like domain and a Db1 homology (DH) domain, and comparisons among ezrin-like domain of human CDEP, ezrin, 20 and band 4.1, in which the residues conserved with respect

to CDEP are indicated in bold type;

Fig. 4 is a view showing comparisons among DH domain of human CDEP, human Db1, rat Ost, mouse Est2, and human FGD1, in which the residues conserved with respect to CDEP are indicated in bold type;

Fig. 5 is a schematic view showing the relation between CDEP and other proteins;

Fig. 6 is an electrophoretogram showing the results of RT-PCR (reverse transcription PCR) of mRNA of type II collagen, aggrecan, cartilage matrix protein (CMP), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (upper row), DEC1 and CDEP (lower row), expressed in response to Bt₂cAMP in fetal human chondrocytes, in which cAMP+ represents the presence of Bt₂cAMP, while cAMP- represents the absence of Bt₂cAMP;

Fig. 7 shows the expression of CDEP, type X collagen, ALP, and aggrecan in each of R, G1, G2 and G3 in Example 4;

Fig. 8 shows the expression of CDEP in each stage of differentiated chondrocytes, and the effect of PTH addition on the expression;

Fig. 9 shows the time courses of CDEP expression after addition of Bt₂cAMP and PTH;

Fig. 10 shows the time course of CDEP expression after addition of PTH;

Fig. 11A shows the results of immunofluorescence

staining (under CNF2(-) conditions) indicating the localization of CDEP in the cell; and

Fig. 11B shows the results of immunofluorescence staining (under CNF2(+) conditions) indicating the localization of CDEP in the cell.

Best Mode for Carrying Out the Invention

(Protein, DNA encoding the protein)

The protein provided by the present invention is derived from DNA discovered by the invention that is set forth in SEQ ID NO: 1 in the sequence listing. A preferred example of the protein has an amino acid sequence set forth in SEQ ID NO: 2 in the sequence listing. As will be described later on in Examples, this sequence was obtained for the first time as a result of search for a gene expressed specifically in human chondrocytes in a differentiated state. The protein having this derived amino acid sequence is called CDEP in the present specification. As will be described below, proteins having different amino acids from those of the above protein, but having substantially the same function as this protein are also collectively called CDEP.

The total nucleotide sequence obtained as the gene encoding the protein CDEP (the gene will be referred to as CDEP cDNA) is described in SEQ ID NO: 1 in the sequence listing. The correspondences with amino acids deduced from the

resulting nucleotide sequence are set forth in SEQ ID NO: 1 in the sequence listing, and Fig. 1 (amino acids are showed as three characters).

CDEP cDNA includes an open reading frame of 3135 bp starting with the first ATG codon existent on the 49th nucleotide. Since an in-frame stop codon is present upstream from the 5'-region (the 34th nucleotide), moreover, the first ATG is defined as an initiation codon. Thus, CDEP is assumed to comprise 1,045 amino acid residues, and its molecular weight is calculated at 118.6 kDa.

The amino acid sequence of CDEP deduced from the above nucleotide sequence is described in SEQ ID NO: 2 in the sequence listing, and Fig. 1. Based on the resulting amino acid sequence, the amino acid sequence of CDEP is found to lack a signal peptide for secretion, and a hydrophobic region of a transmembrane or membrane-bound domain.

At least three polyadenylation signals and polyadenylation sites are found to exist. The total length of 3287 bp, 3306 bp or 3442 bp, excluding the poly(A) tract, is consistent with the size of mRNA (3.5 kb, indicated by an arrow as 3.5 kb in Fig. 2(a)) determined by northern blotting analysis to be described below.

Homology search of the above amino acid sequence by the use of protein database has shown that the CDEP protein includes all of three functional domains, i.e., ezrin-like domain, DH domain, and PH domain (Figs. 1, 3, 4). No protein

has been known which possesses all of ezrin-like domain, DH domain, and PH domain.

The ezrin-like domain that CDEP has refers to the one conserved in a band 4.1 superfamily, including ezrin, radixin, moesin, and band 4.1 that have been known. Fig. 3 shows the homology of the ezrin-like domain of CDEP to them. The ezrin-like domain of CDEP has homology of 27.5% to ezrin, and homology of 43.7% to band 4.1.

A protein such as ezrin is believed to play the role of a linker protein between the cytoskeleton and the cell membrane. Of some proteins recently reported, including an ezrin-like domain in the N-terminal region, merlin is known to be an tumor suppressor molecule which causes neurofibroma type 2, and PTPMEG1 and PTPH1 are known to be members of a cytoplasmic protein tyrosine phosphatase (PTP) family. These proteins are known to take part in the transmission of signals from outside of the cell to the inside of the cell at the boundary between the cell membrane and the cytoskeleton. A notable fact is that ezrin, radixin, and moesin (these are called ERM proteins) bind to a cytoplasmic domain of CD44, a hyaluronate receptor, through their N-terminal region. Also noticeable is that the binding of the ERM proteins to CD44 is enhanced by GTP γ S, and markedly suppressed by C3 toxin, a specific inhibitor of Rho. As noted from these facts, GTP-Rho is required for the formation of a CD44/ERM protein complex.

The CDEP protein of the invention also has the DH domain and the PH domain. The Rho GEF family, including Db1, Ost, Ect2 and Lbc which are oncogene products, and FGD1 which is a causative gene for faciogenital dysplasia, is also known to include a DH domain, and to be bound directly to a PH domain (DH-PH sequence) at the C-terminus. The homology of CDEP in terms of DH domain is 22.9% for Db1, 22.9% for Ost, 22.3% for Ect2, and 25.6% for FGD1 (Fig. 4).

Rho GEF is known to convert GDP-Rho (inactive type), in particular, into GTP-Rho (active type), while Ras GEFs are known to convert GDP-Ras into GTP-Ras. Rho and Ras belong to a small GTP-binding protein superfamily, and are known to take part in intracellular signal transduction. The superfamily is divided into five subfamilies, i.e., Ras, Rho, Rab, Arf, and other. The Rho subfamily (Rho, Rac, and Cdc42) is known to control the morphology, migration, aggregation and adhesion of cells by contraction of actin filaments. It is also known that some regulators of Rho, such as GEFs and GTPase-activating proteins (GAPs), are present upstream in the Rho cascade.

It is further known that the DH-PH domain is necessary for Rho GEF activity, while the Cdc-25 domain is necessary for Ras GEF activity; some of Ras GEF family members, such as Ras-GRF and Sos, have Ras GEF activity, but have no Rho GEF activity, even though they have DH-PH domain in addition to Cdc-25 domain.

As described above, CDEP of the invention is characterized by lacking the Cdc-25 domain and containing the DH-PH domain. Thus, CDEP is one of the members of the Rho GEF family, rather than the Ras GEP family.

5 The ERM proteins having the ezrin-like domain are known to be involved in signal transduction leading to morphological changes of cells through the formation of a CD44/ERM/actin complex. Formation of this complex requires the activity of Rho by Rho GEF. CDEP obtained in the invention is found to be a very unique protein containing an ezrin-like domain and an Rho GEF-like domain in one molecule (Fig. 5). This is suggestive of a possibility that this substance will modify signal transduction in the CD44 path and/or a path mediated by other receptor. Furthermore, many members of the Rho GEF family become oncogenes for lack of the N-terminal region. This finding suggests that CDEP plays an important role in controlling the adhesion, diffusion, migration, proliferation, and differentiation of cells, including chondrocytes.

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20 Therefore, the CDEP protein of the invention includes not only a protein having the amino acid sequence set forth in SEQ ID NO: 2, but also a protein having a novel amino acid sequence comprising the amino acid sequence of SEQ ID NO. 2 in which one to several amino acids have been deleted, substituted or added, the novel amino acid sequence having
25 an ezrin-like domain, a DH domain, and a PH domain in the

molecule.

Particularly, the CDEP protein includes a protein in which an amino acid sequence ranging from the 1st to 373rd amino acids in SEQ ID NO: 2 (called ezrin-like domain) has
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homology of 70% or more (preferably 85% or more) to the amino acid sequence of the SEQ ID NO: 2, in which an amino acid sequence ranging from the 544th to 737th amino acids in SEQ ID NO: 2 (called DH domain) has homology of 70% or more (preferably 85% or more) to the amino acid sequence of the
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SEQ ID NO: 2, and in which an amino acid sequence ranging from the 764th to 854th amino acids in SEQ ID NO: 2 (called PH domain) has homology of 70% or more (preferably 85% or more) to the amino acid sequence of the SEQ ID NO: 2.

The protein having such amino acid sequences has
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substantially the same domains as ezrin-like, DH and PH domains characteristic of CDEP, and is thus presumed to have substantially the same biochemical functions as does CDEP.

The CDEP protein, which has a different amino acid sequence from that of CDEP having the amino acid sequence
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described in SEQ ID NO: 2 in the sequence listing and which has substantially the same functions, can be obtained by a method for replacing, deleting, or inserting particular amino acid residues in the amino acid sequence described in SEQ ID NO: 2 in the sequence listing. Such a method is
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not restricted, and a publicly known method can be used preferably as this method. For example, the CDEP protein

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can be formed by introducing a mutation, such as substitution, deletion or insertion of nucleotide, into the nucleotide sequence by a publicly known method such as site-directed mutagenesis, and people skilled in the art can easily select the type of the mutant protein. That is, derivatives having functions comparable to those of particular proteins can be prepared by any methods of chemical peptide synthesis well known in the art, or ordinary genetic engineering methods, for instance, recombinant DNA technologies based on DNA sequence as disclosed in Sambrook et al., Molecular Cloning, Cold Spring Harbour Laboratory Press, New York, USA, Second Edition, 1989. Concretely, the method described, for example, in H. Neurath R.L. Hill, "The Proteins" (Academic Press, New York, 1979, especially in Fig. 6 on page 14) is usable. Substitutions that occur most usually are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu and Asp/Gly, and vice versa.

Thus, DNA encoding the CDEP protein includes not only that coding for a protein having the amino acid sequence set forth in SEQ ID NO: 2, but also DNA encoding a protein having a novel amino acid sequence comprising the amino acid sequence of SEQ ID NO. 2 in which one to several amino acids have been deleted, substituted or added, the novel amino acid sequence having an ezrin-like domain, and DH and PH domains in the molecule.

Particularly, the above DNA includes DNA encoding a protein in which an amino acid sequence ranging from the 1st to 373rd amino acids in SEQ ID NO: 2 (ezrin-like domain) has homology of 85% or more to the amino acid sequence of the SEQ ID NO: 2, in which an amino acid sequence ranging from the 544th to 737th amino acids in SEQ ID NO: 2 (DH domain) has homology of 85% or more to the amino acid sequence of the SEQ ID NO: 2, and in which an amino acid sequence ranging from the 764th to 854th amino acids in SEQ ID NO: 2 (PH domain) has homology of 85% or more to the amino acid sequence of the SEQ ID NO: 2.

The DNA "encoding or coding for a protein" refers to DNA, if double-stranded, in which one of the complementary two strands has a nucleotide sequence coding for the protein. The DNA of the invention further includes DNA comprising a nucleotide sequence ranging from the 49th to 3183rd bases in the nucleotide sequence set forth in SEQ ID NO: 1, or a nucleotide sequence complementary thereto.

The invention also includes a DNA sequence encoding the CDEP protein, a DNA sequence including a strand complementary to the above sequence, or these sequences, a DNA sequence hybridized under standard conditions with the sequence or a fragment derived therefrom, and a DNA sequence which is not hybridized under standard conditions with the DNA sequence because of degeneracy of the genetic code, but which encodes a polypeptide having exactly the

same amino acid sequence.

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5 The "standard conditions" for hybridization refer to conditions generally used by people skilled in the art to detect specific hybridization signals, and those conditions mentioned, for example, in Sambrook et al., Molecular Cloning, Cold Spring Harbour Laboratory Press, New York, USA, Second Edition, 1989, preferably, so-called stringent hybridization-non-stringent washing conditions known among people skilled in the art and stated by Sambrook et al., more preferably, so-called stringent hybridization-stringent washing conditions. The stringent conditions preferably used in the invention are conditions under which a so-called specific hybrid is formed and a non-specific hybrid is not formed. It is difficult to express these conditions clearly as numerical values. However, concrete conditions include a temperature in the range of from the T_m of a hybrid of nucleic acids with high homology, e.g., a hybrid of completely matching nucleic acids, to a temperature 20°C lower than the T_m , or conditions at which DNA's having homology of 80% or more are hybridized, but nucleic acids having lower homology are not hybridized.

20 Furthermore, it is included in the invention to provide a DNA sequence which can be prepared by polymerase chain reaction (PCR) with the use of primers, designed based on the DNA sequence of SEQ ID NO: 1 of the sequence listing disclosed in the present specification, in accordance with

the method well known in the art.

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The DNA according to the invention can be synthesized on the basis of one of the nucleotides sequences of the DNA that have been determined by the invention as shown in Examples to be offered later on. The DNA can also be obtained from chromosomal DNA by PCR or hybridization using oligonucleotide primers or a probe prepared based on the nucleotide sequence. Alternatively, the DNA can be obtained by performing RT-PCR using cartilage mRNA, or by screening a cDNA library of cartilage or the like with the use of a polynucleotide, which has a nucleotide sequence encoding all or part of CDEP protein, as a probe.

The CDEP protein of the invention can be produced by inserting the aforementioned DNA of the invention into a publicly known expression vector to construct a recombinant plasmid, introducing this recombinant plasmid into a cell to obtain a transformant, culturing the transformant in a suitable medium to form and accumulate the protein of the invention in the culture, and collecting the protein from the culture.

As the cell and expression vector, a host-vector system usually used in the expression of a foreign protein can be used. Examples are combinations of procaryotic cells, such as *Escherichia coli*, and suitable expression vectors, and combinations of eucaryotic cells, such as mammalian cells, and suitable expression vectors. The culture media and

culture conditions are suitably selected in harmony with the cells used.

The protein of the invention may be expressed as a fusion protein combined with other protein. Moreover, the protein of the invention may be expressed with its entire length, or may be expressed partially as a partial peptide.

The culture refers to a culture medium, and cells in the culture medium. Collection of the protein of the invention from the culture can be performed by a publicly known protein purification method using the activity, etc. of the protein of the invention as indicators.

If desired, the protein of the invention can be isolated from a heterogenetic organism by using the aforementioned nucleotide sequence information (in consideration of the codons used), by colony hybridization or southern blot hybridization using a probe synthesized in accordance with an amino acid sequence deduced from the nucleotide sequence, or by polymerase chain reaction (PCR) using primers synthesized by the information.

(Antibody)

An antibody which can bind to the protein of the invention (hereinafter referred to as the antibody of the invention) can be prepared by the customary method using the protein of the invention as an antigen. The antibody of the invention may be polyclonal or monoclonal.

The protein of the invention may be used, unchanged,

as an antigen, but is preferably used as an antigen after being joined to a carrier protein, such as keyhole limpet hemocyanin, bovine serum albumin, or ovalbumin, and/or in combination with an adjuvant.

5 The polyclonal antibody can be obtained by immunizing an animal, such as mouse, rabbit, guinea pig or sheep, with intraperitoneal or intravenous injection of the antigen, and taking the serum from the immunized animal.

10 The monoclonal antibody can be obtained, for example, in the following manner: An animal, such as mouse, rabbit, guinea pig or sheep, is immunized with subcutaneous, intraperitoneal or intravenous injection of the antigen. Then, the spleen or lymph node is removed, and a cell is taken therefrom. The cell is fused with a myeloma cell derived from an animal, preferably, allogeneic to the immunized animal to establish a hybridoma. From the resulting hybridoma, a cell strain which continuously produces specific antibodies to the antigen is chosen by repeating screening and cloning. The so selected cell strain is cultured in a suitable medium, and monoclonal antibodies are produced in the medium. Alternatively, the cell strain is cultured in vivo, e.g. intraperitoneally in the mouse, to produce monoclonal antibodies in ascitic fluid or the like.

20 Methods for purification of the resulting polyclonal and monoclonal antibodies include, for example, salting out by ammonium sulfate, ion exchange chromatography using a

DEAE cellulose column or the like, affinity chromatography using a protein A column or the like, and immunoadsorbent chromatography. The antibody of the invention can be detected, for example, by immunoassay using the protein of the invention and a labeled antibody.

The antibody of the invention may be a fragmented one as long as the antigen-binding site (Fab) is conserved. An example of the fragmented antibody is an Fab-containing fragment obtained by decomposing the antibody with a protease which does not decompose the antigen-binding site, such as papain.

The antibody of the invention may have been labeled by being bound to a labeling substance. The labeling substance is not restricted, if normally usable for labeling a protein. Its examples are enzymes, isotopes, and fluorescent materials.

A method according to the invention for screening a regulator of cell differentiation induction using the protein of the invention may, for example, comprise adding the substance in question to a cell having differentiation potency, such as tetracarcinoma, and investigating what actual changes occurred in the cell, for instance, whether the cell differentiated, based on the shape of the cell and/or the expression of a marker gene. Alternatively, the screening method of the invention may be to add various medicines, which have been chemically synthesized, to the

cell, and search for agents which can change the amount of expression of CDEP.

(Kit for detection of differentiated chondrocytes, Method for detection of differentiated chondrocytes)

5 The method of the invention for detection of differentiated chondrocytes uses the aforementioned gene of the invention which is specifically expressed in differentiated chondrocytes.

10 Concretely, (a) the CDEP protein of the invention is used in a publicly known method to enable differentiated chondrocytes to be specifically detected. Alternatively, (b) the DNA of the invention, or its derivative is used in a publicly known method to enable differentiated chondrocytes to be specifically detected. Further alternatively, (c) the
15 antibody of the invention is used in a publicly known method to enable differentiated chondrocytes to be specifically detected. Any combination of these methods can be used as the method of the invention.

20 (Method for screening a regulator of cell differentiation induction, Kit for screening a regulator of cell differentiation induction)

25 The method of the invention for screening a regulator of cell differentiation induction uses the aforementioned gene of the invention which is specifically expressed in a differentiated chondrocytes.

Concretely, (a) the CDEP protein of the invention is

used in a publicly known method to enable a regulator of cell differentiation induction to be screened.

Alternatively, (b) the DNA of the invention is used in a publicly known method to enable a regulator of cell

5 differentiation induction to be screened. Further alternatively, (c) the CDEP protein of the invention is used in a publicly known method to enable a regulator of cell differentiation induction to be screened. Any combination of these methods can be used as the method of the invention.

10 More concretely, the method for screening an anti-tumor agent as a regulator of cell differentiation induction may, for example, be to add various chemically synthesized agents to a cell cancerized by introduction of the aforesaid CDEP cDNA deficient on the N-terminal side, and search which of the agents return the cell to normal.

15 The invention further includes a regulator of cell differentiation induction which can be obtained by the above screening method.

(Biological functions of CDEP)

20 The biological functions of CDEP obtained by the invention are summarized here. CDEP has an ezrin-like domain, and DH and PH domains in one molecule. In this view, CDEP may work by the following mechanism: The Ezrin-like domain dissociates Rho GDI from Rho, and then the DH and PH domains perform GDP-GTP exchange reaction in one molecule to regulate the activity of Rho, thereby controlling cell shape. Hence,

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CDEP is considered to be a very advantageous factor for activation of Rho among Rho GEFs.

Examples

5 The present invention will now be described with reference to Examples to be offered below. PCR primers used in the Examples were synthesized by an ordinary automatic method of synthesis.

Sub 10 B For type II collagen: CATACCGGTAAGTGGGGCAAGACTG,

TGCCCAGTTCAGGTCTCTTA

For aggrecan: TGCTACTTCATCGACCCCAT, AAAGACCTCACCTCCATCT

For CMP: GTCGATTACGTGGAGAGCTA, ATGAACTTCTTCACCAGCTC

For GAPDH: GTCAAGGCTGAGAACGGGAA, TCCACCACCCTGTTGCTGTA

For DEC1: ATCAGACCCAGCTCCCAAAG, CACAGACCCAGCTCCCAAAC

15 For CDEP: CCTTCAGGAAAACTCGTGTC, TTGGAGTTGTGTGTGGTCAG

For CDEP cDNA fragment: GCCAAAATAGTCACCTTCCACGAGG,

CCTTCAGGAAAACTCGTGTC, AAACGRAAGAAYGTRTGRTGYTCWACACA,

TTCCAGCTCCTAGAGATTGC, TCGTCTTCGCTCTCCTCAAT,

20 CGGGTAACAACAGGCGGACGGA

(Example 1)

Culture of differentiated chondrocytes was performed in the following manner in accordance with the method described in Biochemical and Biophysical Research

25 Communications 236, 294-298 (1997), M. Shen, T. Kawamoto, W. Yan, K. Nakamasu, M. Tamagami, Y. Koyano, M. Noshiro,

and Y. Kato:

From a human fetus spontaneously aborted at about 25 weeks of pregnancy (obtained from Norman Bethune University of Medical Sciences), an epiphysical cartilage on the femur side of the knee joint was obtained. From this cartilage, chondrocytes were isolated by the same method as described in Simomura et al. (1975), *Calcif. Tissue Res.* 19, 179-187, except that cartilage slices were incubated for 3 hours in an α -modified Eagle's medium (α -MEM) containing 3 mg/ml of collagenase (type IA, Sigma). The obtained cells were seeded on type I collagen coated dishes at a density of 1×10^5 cells/dish. The cells were cultured in α -MEM (10 ml/dish) containing 10% bovine fetal serum, 50 μ g/ml ascorbic acid, 32 units/ml of penicillin, and 40 μ g/ml of streptomycin. When the cells became subconfluent, dibutyryl cAMP (dbcAMP) (1 mM) was added to the culture medium. The cells were cultured for 2 weeks or more in the presence and absence of dbcAMP, and then morphological observation of the cells was made. Also, the cells were harvested, fixed with ethanol, and stained with toluidine blue.

The chondrocytes cultured in the presence of dbcAMP took a spherical form, and were rich in extracellular matrix. Whereas the chondrocytes cultured in the absence of dbcAMP were spindle-shaped, similar to fibroblasts, and deficient in extracellular matrix.

Staining with toluidine blue, which selectively

stains sulfated proteoglycan, satisfactorily stained the chondrocytes cultured in the presence of dbcAMP, but scarcely stained the chondrocytes cultured in the absence of dbcAMP.

The expression of mRNA of type II collagen, aggrecan and CMP as molecule markers was examined by RT-PCR. The expression of the molecule marker in the presence of dbcAMP was compared with that in the absence of dbcAMP. It was suggested tat a differentiated state was maintained in the presence of dbcAMP (Fig. 6).

Thus, the chondrocytes cultured in the presence of dbcAMP were admitted to maintain a differentiated state as cartilage, namely, a differentiated phenotype.

The above effect of dbcAMP was examined for dose dependency. This effect was found to increase dose dependently, and peaked at 0.3 to 0.5 mM.

Chondrocytes were cultured in the same manner as described above, except that bFGF (0.4 ng/ml) and TGF- β (3 ng/ml) were used instead of dbcAMP. bFGF and TGF- β are reported to stabilize or stimulate the expression of the differentiated phenotype of rabbit and chicken chondrocytes. The expression of the differentiated phenotype of the human-derived chondrocytes was not maintained with the use of bFGF and TGF- β .

(Example 2) Gene specifically expressed in chondrocytes in a differentiated state

From the chondrocytes cultured in the presence of
dbcAMP and in the absence of dbcAMP as described in Example
1, total RNA was extracted by the guanidine
thiocyanate/cesium trifluoroacetate method. Poly(A)⁺RNA
5 was concentrated using Oligotex-dT30 (Roche). Clones
encoding mRNA, which is expressed in differentiated
chondrocytes (+dbcAMP), but not in dedifferentiated
chondrocytes (-dbcAMP), were selected by subtractive
hybridization. That is, cDNA derived from mRNA of the
10 differentiated chondrocytes was hybridized with an excess
amount of cDNA derived from mRNA of the dedifferentiated
chondrocytes, by use of PCR Select cDNA Subtraction Kit
(Clontech). cDNA which is not hybridized, namely, which is
expressed in a differentiated state, was amplified by
15 suppression PCR. The resulting PCR product was cloned into
a T tail vector pGEM-T (Promega), and nucleotide sequencing
of about 120 clones was performed. For further analysis,
one clone was selected, and the corresponding protein product
was named CDEP.

20 Fig. 2A and Fig. 2B show the results of northern
blotting analysis of CDEP mRNA expression in various human
fetal tissues by the use of a 1.6 kb fragment of CDEP as
the probe. Here, the transcript of 5.0 kb was also detected
by northern blotting analysis. This finding suggests that
25 in addition to the PCR product derived from 3.5 kb mRNA,
a larger band induced from 5.0 kb mRNA probably hybridizable

with the CDEP probe was found in RACE-PCR as well. However, the nucleotide sequencing of the product corresponding to the transcript of 5.0 kb has not been performed yet.

The expression pattern of CDEP mRNA in cultured chondrocytes and various human tissues by northern blotting analysis showed that two main bands, 3.5 kb and 5.0 kb, were detected in cyclic AMP dependent differentiated chondrocytes, as illustrated in Fig. 2A, but they were not detected in fibroblasts without cyclic AMP. In fetal tissues, the highest level of CDEP mRNA was observed in the brain and the spleen, and low to intermediate levels of CDEP mRNA were noted in the heart, liver and intestine (Fig. 2A). In adult tissues, the amount of expression was intermediate in the kidney, testis, and lung, and considerably small in the heart, liver and intestine (Fig. 2B).

Northern blotting was performed in the following manner: The total RNA (10 μ g) was electrophoresed on a 1% agarose gel containing formaldehyde, and transferred onto Hybond-N Membrane (Amersham).

Fetal human chondrocytes cultured in the presence and absence of Bt2cAMP, various fetal human tissues (Fig. 2A), and adult tissues (Fig. 2B) were electrophoresed on a 1% agarose gel containing formaldehyde, and transferred onto a nylon membrane. The membrane was hybridized with a CDEP probe (CDEP) or a glyceraldehyde-3-phosphate dehydrogenase probe (GAPDH). To examine the tissue distribution, total

RNA of various fetal human tissues were supplied by Dr. Li Yu of Norman Bethune University of Medical Sciences. A 1.6 kb fragment of CDEP was labeled with [³²P]dCTP, and used as a hybridization probe. The membrane was washed with 0.2 X SSC containing 0.5% SDS for 30 minutes at 65°C. A biomax X-ray film was exposed to light through the washed membrane using a sensitizing film at -70°C.

Nucleotide sequencing of the full length of CDEP cDNA was performed in the following manner: CDEP full length cDNA was isolated by the rapid amplification cDNA ends (RACE) method using Marathon cDNA Amplification Kit (Clontech). In detail, double-stranded cDNA was joined to Marathon cDNA adaptor, and subjected to suppression PCR. The reaction was carried out using an adaptor primer, and a gene specific primer designed for CDEP on the basis of the nucleotide sequence of CDEP. The amplified cDNA sample was separated using a 4% polyacrylamide gel, and DNA of major bands was extracted from the gel and subcloned into pGEM-T. The subcloned plasmid double-stranded DNA and a series of synthetic oligonucleotides were used as a sequencing template and specific primers, respectively. DNA sequencing was performed by the Sanger method using Sequenase 7-deaza-dGTP DNA Sequencing Kit (Amersham), or ABI PRISM 310 Autosequencer (Perkin Elmer).

The so determined nucleotide sequence of CDEP cDNA and an amino acid sequence deduced therefrom are set forth

in SEQ ID NO: 1. Only the amino acid sequence is set forth in SEQ ID NO: 2. CDEP cDNA has an open reading frame of 3135 bp. The length of 3287 bp, 3306 bp or 3442 bp, excluding the poly(A) region, agrees highly with the size (3.5 kb) of the mRNA obtained by the aforementioned northern blotting analysis. Since an in-frame stop codon exists in the 5'-region (nucleotide 34), the first ATG is admitted as an initiation codon.

10 (Example 3) Expression of CDEP mRNA in rabbit costal
cartilage growth plate slices
15 1 Total RNA was extracted from each of layers, i.e.,
costal cartilage growth plate slices (a portion near the
bone side was divided into three parts designated as G3,
G2 and G1) and a resting cartilage layer (called R) of a
4-weeks-old male Japanese albino rabbit. The extract was
examined for the expression level of CDEP mRNA by the RT-PCR
method. The expression levels of type X collagen, alkaline
phosphatase, and proteoglycan aggrecan, differentiation
20 markers of chondrocytes, in the respective layers were
confirmed by RT-PCR.

Extraction of total RNA was performed from the
cartilage tissue by guanidine-cesium chloride
ultracentrifugation. The RT-PCR method used SUPERScript
25 Preamplification System (GIBCOBRL). For DNA amplification,
DNA Thermal Cycler (Perkin-Elmer Cetus) was used.

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B2 Reverse transcription was performed using the total RNA (0.5 μ g) extracted from the cartilage tissue, and amplification was carried out using an upstream specific primer (5'-TCACTTCGTGGTTTCAGAGC-3') and a downstream specific primer (TCGTCTTCGCTCTCCTCAAT-3') that were designed based on the nucleotide sequence of CDEP to obtain a CDEP cDNA fragment. The conditions for the amplification reaction were set to carry out a denaturation reaction (95°C, 1 min), annealing and extension (65°C, 3 min), and 20 cycles of amplification were performed. The resulting DNA was electrophoresed for 15 minutes at 100 V on a 1% agarose gel.

PCR southern blotting was performed in the following manner: The electrophoresed DNA in the gel was denatured in a 0.5N NaOH solution containing 1.5M NaCl, and then transferred onto a nylon membrane Nytran0.45 (Schleicher & Schuell) with the use of 6X SSC. This membrane was subjected to a hybridization reaction for 16 hours at 68°C in a hybridization solution containing a [³²P]labeled CDEP probe labeled with Oligolabelling Kit (Pharmacia Biochem). After the reaction, the membrane was washed, and autoradiography was performed. The radioactivity was measured with BAS2000 Image Analyzer (Fujix).

Rabbit costal cartilage growth plate chondrocytes were cultured in the following manner: The growth plates were separated from the costal cartilages of the 4-weeks-old male Japanese albino rabbit. They were digested with 0.1%

EDTA, 0.1% trypsin containing PBS and 0.05% collagenase containing DMEM, and chondrocytes were isolated. Six-well tissue culture plates were seeded with 300,000 cells per well, and the cells were cultured with alpha MEM containing 10% bovine fetus serum. The culture medium was replaced at intervals of 2 days, starting on the 5th day of culture.

The expression of CDEP mRNA in the rabbit costal cartilage growth plate chondrocytes culture system was investigated by extracting total RNA from the cell layer on the 6th, 10th, 14th, 18th, 22nd and 26th days of culture, and determining the expression level of CDEP mRNA by RT-PCR.

The induction of CDEP mRNA by PTH in the rabbit costal cartilage growth plate chondrocytes culture system was investigated by adding PTH (1-84) ($1 \times 10^{-7} \text{M}$) after 6, 10, 14, 18, 22 and 26 days of culture, incubating the system for a further 24 hours, then extracting total RNA, and determining the expression level of CDEP mRNA at each stage by RT-PCR.

The time course of the induction of CDEP mRNA expression by PTH and cAMP was investigated by adding PTH (1-84) ($1 \times 10^{-7} \text{M}$) and Bt_2cAMP in rabbit costal cartilage growth plate chondrocytes on the 12th day of culturing, extracting total RNA 1, 3, 6, 12 and 24 hours later, and determining the expression level of CDEP mRNA at each time point by RT-PCR.

The results of the foregoing experiments and the results given below (Example 4) gave the following findings:

(1) In the in vivo cartilage tissue, more CDEP is expressed

in the hypertrophic layer showing the expression of type X collagen and ALP, the markers of terminal differentiation of chondrocytes (hypertrophy, calcification) (Fig. 7, Fig. 11A, Fig. 11B). (2) In the in vitro chondrocytes culture system, more CDEP mRNA is expressed in the hypertrophic stage and later (on the 22nd day of culturing and later), and the amount of expression is further increased upon the addition of PTH using cAMP as a second messenger (Fig. 8). Furthermore, (3) the level of CDEP mRNA rises transiently at a very early stage (as early as 1 hour later) in short-term induction during the period of from 0 to 6 hours after addition of cAMP and PTH, and then rises over time (Fig. 9). Moreover, the expression in the period of from 0 to 48 hours after addition of PTH becomes biphasic (Fig. 10).

(Example 4) Intracellular localization of CDEP by immunofluorescence staining

Immunofluorescence staining was performed to investigate the localization of CDEP in the cell with the use of MRC5, a human lung fibroblast cell line. That is, 3,000 cells were seeded on a 1x1 cm cover glass, and cultured for 24 hours in DMEM. Then, cytotoxic necrotizing factor 2 (CNF2) derived from enteropathogenic E. coli was added, and the so treated cells were cultured for a further 12 hours together with a CNF2-free control group. The cultures were washed with PBS, and then fixed with 2% paraformaldehyde

for 30 minutes at room temperature. Then, the system was washed with PBS again, treated for 3 minutes with 0.5% Triton X-100, and further washed with PBS. Then, the system was incubated for 1 hour in PBS containing 5% sheep serum at room temperature to block nonspecific binding. After washing with PBS containing 0.05% Tween 20 (PBST), the system was incubated for 2 hours at room temperature along with anti-CDEP antiserum diluted 1:100 with PBS. Then, after washing with PBST, the system was incubated for 30 minutes at room temperature under light-shielded conditions together with FITC-labeled anti-rabbit sheep IgG (Cappel) diluted 1:1000. After washing with PBST, the system was observed with a confocal laser microscope (LSM410, Carl Zeiss) (Figs. 11A, 11B).

In the absence of CNF2, the cytoplasm was stained as a whole. In the presence of CNF2, polymerization of actin took place. CDEP was localized in filamentous processes and ruffling sites of the cell membrane that occurred on this occasion.

Industrial Applicability

As described above, the present invention provides genes expressed specifically in differentiated human-derived chondrocytes. These genes are important not only in analyzing the mechanism of differentiation or degeneration of a cartilage, but also in developing gene

therapies for osteoarthritis and rheumatoid arthritis. Furthermore, CDEP according to the invention is expressed in cartilage and other tissues, and thus its important actions in such other tissues are suggested.

5 That is, CDEP of the invention, in view of its structural features, serves as a regulatory factor for a cytoskeleton binding to protein on a cell membrane, and also has a structure characteristic of an epistatic factor for regulating the functions of Rho. By controlling an
10 actin-series cytoskeleton, i.e., activating Rho in response to signals from upstream to control signal transduction downstream, CDEP may maintain cell shape or regulate cell adhesion, cell aggregation, or cell motility. From the aspect of being a regulatory factor for cytoskeleton, CDEP
15 may play important roles in the reconstruction of a cytoskeleton which occurs as a result of stimulation by cell growth factors, or cell transformation. For chondrocytes, changes in cell shape are closely related to differentiation, and it is known that differentiated chondrocytes takes a
20 spherical form, while dedifferentiated chondrocytes is spindle-shaped. Hence, if the shape of a cell can be changed to a spherical form by accelerating or suppressing the functions of CDEP, it may be possible to induce or maintain the differentiation of chondrocytes. Gene introduction with
25 the expectation of such an action may make it possible to control the differentiated state of chondrocytes with

deviating functions (dedifferentiated chondrocytes) in arthropathies, including osteoarthritis.

Among other Rho GEF family members, some are known to work as oncogenes by arousing such abnormalities as the lack of an amino acid on the N-terminal side. They are reported to enhance the infiltrating ability of cancer. If it is found that active type mutation, such as deficiency in part of a CDEP gene, causes canceration of a cell, a novel therapeutic drug with a specific suppressive effect may be developed with CDEP as a target.

Also, it is conceivable to prepare a dominant negative mutant of CDEP, and introduce its gene into a cancer cell to inhibit the activity of the cancer cell, thereby achieving cancer treatment. In other words, CDEP is likely to be useful in elucidating the mechanism of control of the functions of the entire Rho family.

Ezrin binds to CD44, etc. on a cell membrane at its N-terminus, and binds to actin on its C-terminal side, thereby taking part in cytoskeletal regulation. Rho is considered to regulate this binding. CDEP, which has an ezrin-like domain at the N-terminal, and DH and PH domains at the C-terminal, may bind to protein on the cell membrane at the N-terminal, and activate Rho on the C-terminal side. Thus, CDEP may be a regulatory factor for a cytoskeleton which binds to a cell membrane, and also be a factor for regulating signals to Rho by feedback of the state of the cytoskeleton.